



INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

<p>(51) International Patent Classification ⁵ : A61L 27/00</p>	<p>A1</p>	<p>(11) International Publication Number: WO 93/20858 (43) International Publication Date: 28 October 1993 (28.10.93)</p>
<p>(21) International Application Number: PCT/EP93/00933 (22) International Filing Date: 16 April 1993 (16.04.93) (30) Priority data: PD92A000072 17 April 1992 (17.04.92) IT (71) Applicant (for all designated States except US): FIDIA S.P.A. [IT/IT]; Via Ponte della Fabbrica, 3/A, I-35031 Abano Terme (IT). (72) Inventors; and (75) Inventors/Applicants (for US only) : DORIGATTI, Franco [IT/IT]; Via Segantini, 23, I-38015 Lavis (IT). CALLEGARO, Lanfranco [IT/IT]; Via Bravi, 35, I-35020 Ponte di Brenta (IT). ROMEO, Aurelio [IT/IT]; Viale Ippocrate, 93, I-00161 Rome (IT).</p>		<p>(74) Agent: VOSSIUS & PARTNER; Siebertstrasse 4, P.O. Box 86 07 67, D-8000 Munich 86 (DE). (81) Designated States: AU, BB, BG, BR, CA, CZ, FI, HU, JP, KP, KR, LK, MG, MN, MW, NO, NZ, PL, RO, RU, SD, SK, UA, US, VN, European patent (AT, BE, CH, DE, DK, ES, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG). Published <i>With international search report. Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.</i></p>
<p>(54) Title: BIOMATERIALS FOR BONE REPLACEMENTS (57) Abstract Bonding solutions for granular bone replacements are provided, comprising hyaluronic acid or hyaluronic acid derivatives such as hyaluronic acid esters and hyaluronic acid antibiotic salts in combination with natural or artificial bone granules. These solutions can be used in human and veterinary dentistry and bone surgery as granular bone replacements to promote the growth and repair of damaged or defective bone tissue.</p>		

BEST AVAILABLE COPY

FOR THE PURPOSES OF INFORMATION ONLY

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AT	Austria	FR	France	MR	Mauritania
AU	Australia	GA	Gabon	MW	Malawi
BB	Barbados	GB	United Kingdom	NL	Netherlands
BE	Belgium	GN	Guinea	NO	Norway
BF	Burkina Faso	GR	Greece	NZ	New Zealand
BG	Bulgaria	HU	Hungary	PL	Poland
BJ	Benin	IE	Ireland	PT	Portugal
BR	Brazil	IT	Italy	RO	Romania
CA	Canada	JP	Japan	RU	Russian Federation
CF	Central African Republic	KP	Democratic People's Republic of Korea	SD	Sudan
CG	Congo	KR	Republic of Korea	SE	Sweden
CH	Switzerland	KZ	Kazakhstan	SK	Slovak Republic
CI	Côte d'Ivoire	LJ	Liechtenstein	SN	Senegal
CM	Cameroon	LK	Sri Lanka	SU	Soviet Union
CS	Czechoslovakia	LU	Luxembourg	TD	Chad
CZ	Czech Republic	MC	Monaco	TG	Togo
DE	Germany	MG	Madagascar	UA	Ukraine
DK	Denmark	ML	Mali	US	United States of America
ES	Spain	MN	Mongolia	VN	Viet Nam
FI	Finland				

5

10

BIOMATERIALS FOR BONE REPLACEMENTS

BACKGROUND OF THE INVENTION

15 Field of the Invention

The present invention relates to new biomaterials, i.e., bonding solutions for granular bone replacements and bone replacements in the form of pastes, comprising hyaluronic acid and hyaluronic acid derivatives.

20

Description of Related Art

Hyaluronic acid

25 Hyaluronic acid is a natural polysaccharide composed of alternating residues of D-glucuronic acid and N-acetyl-D-glucuronic acid. It is a linear polymer with a wide molecular weight range, depending on the source from which it is obtained, how it is prepared, and how the molecular weight is determined. In nature,
30 it is present in the pericellular gel, in the fundamental substance of the connective tissues of vertebrates, of which it is the main component, in the synovial fluid of joints, in the vitreous humor, in umbilical cord tissues, and in the combs of domestic
35 fowl.

Specific hyaluronic acid fractions with definite molecular weights are known which do not possess inflammatory activity, and which can therefore be used to enhance wound healing or substitute for endobulbar fluids, or which can be used in therapy for joint pathologies by intraarticular injection, as described in European Patent No. 0138572, granted to Applicants.

Also known are hyaluronic acid esters wherein all or part of the carboxy groups of the acid are esterified, and their use in the fields of pharmaceuticals, cosmetics, and biodegradable plastic materials, as described in U.S. Patents Nos. 4,851,521 and 4,965,353 also granted to Applicants.

It is known that hyaluronic acid plays a fundamental role in tissue repair processes, especially in the first stages of granulation tissue formation, stabilizing the coagulation matrix and controlling degradation, favoring the recruitment of inflammatory cells such as polymorphonucleocytes and monocytes, of mesenchymal cells such as fibroblasts and endothelial cells, and directing the subsequent migration of epithelial cells.

It is known that the application of hyaluronic acid solutions to bedsores, wounds and burns accelerates healing. The role of hyaluronic acid in the various stages of tissue repair has been described, by constructing a theoretical model, by Weigel P. H. et al.: "A model for the role of hyaluronic acid and fibrin in the early events during the inflammatory response and wound healing," J. Theor. Biol., 119: 219, 1986.

GRANULAR BONE REPLACEMENTS

Granular bone replacements have been widely studied and used in dentistry and medicine owing to their biocompatible and osteoconductive properties, and because they easily fill cavities of various forms, such as those caused by alveolar bone shrinkage, postextraction cavities, and cystic cavities. The main difficulty when using this material is caused by its lack of binding properties, so that it easily becomes dislodged either during or after application. To overcome this drawback, various types of bonding materials have been proposed with which to prepare pastes containing bone granules. Fibrin is already being used, as described in patent applications JP A 60254640 and JP A 60254641.

One disadvantage of using fibrin as a bonding agent is that it can cause infection by the hepatitis virus, as well as by H.I.V. and other viruses, due to its human origin. Alternative materials have therefore been proposed, such as pullulan, chitin, glycol, carbomethylene chitin, and pectin, as described in patent application EP 0416398.

SUMMARY OF THE INVENTION

An object of the present invention is to provide a viscous solution composed of hyaluronic acid and/or hyaluronic acid esters or salts of hyaluronic acid in association with antibiotics, used singly or in combination, to bind bone replacements in granular form for use in dentistry or surgery of all kinds. Such solutions have excellent biocompatible and bioabsorbable characteristics, and are not liable to cause problems such as infection.

Another object of the present invention is to provide a paste comprising a viscous solution of hyaluronic acid and/or hyaluronic acid esters or salts of hyaluronic acid in association with antibiotics, used
5 singly or in combination, and bone granules. The granules incorporated in the paste are firmly adhered together, thus facilitating their use in dentistry and bone surgery.

Further scope of the applicability of the present
10 invention will become apparent from the detailed description provided below. However, it should be understood that the detailed description and specific examples, while indicating preferred embodiments of the invention, are given by way of illustration only, since
15 various changes and modifications within the spirit and scope of the invention will become apparent to those skilled in the art from this detailed description.

DETAILED DESCRIPTION OF THE INVENTION

20 The following detailed description is provided to aid those skilled in the art in practicing the present invention. Even so, the following detailed description should not be construed to unduly limit the present invention, as modifications and variations in the
25 embodiments herein discussed may be made by those of ordinary skill in the art without departing from the spirit or scope of the present inventive discovery.

The contents of each of the references cited herein are incorporated by reference in their entirety.

30 The foregoing objects are achieved by dissolving hyaluronic acid, esters of hyaluronic acid, or salts of hyaluronic acid in association with antibiotics, used singly or in combination, in water, to form highly viscous solutions. The viscosity of such solutions is at
35 least 15 Pa.s, preferably over 22 Pa.s, at a temperature

of 25°C and 50% \pm 5% relative humidity. The nearer the viscosity to the lower limit, the more liquid the solution; the greater the viscosity, the denser the solution. The correct degree of viscosity for ideal
5 working conditions is a subjective parameter which depends on the individual, who can alter its viscosity by varying the concentration of the solution. The properties of the material comprising the solution, such as its molecular weight, will be selected according to
10 the required degree of viscosity.

The solution according to the present invention is prepared by solubilizing hyaluronic acid, the partial ester of hyaluronic acid or a mixture of the same, or a salt of hyaluronic acid in association with an
15 antibiotic or a mixture of the same, previously sterilized with gamma rays, in sterile water or buffer.

The esters of hyaluronic acid that can be used in the present invention are described in U.S. Patents Nos. 4,851,521 and 4,965,353, and in PCT publication WO 92-
20 13579. the salts of hyaluronic acid in association with antibiotics that can be used in the present invention are described in U.S. Patent No. 5,166,331. These can be used singly or in various combinations with one another, or with hyaluronic acid.

25 The powder is placed in the solubilization vessel under a sterile hood at a temperature of 25°C \pm 2°C and 50% \pm 5% humidity. Solubilization can be achieved in a mixer composed of two spiral elements turning in opposite directions.

Operative conditions depend on the desired viscosity and can be as follows:

5	FINAL VISCOSITY	SOLUBILIZATION TEMPERATURE	NO. TURNS IN MIXER	SOLUBILIZATION DURATION
	15 Pa.s	30°C ± 5°C	60 rpm	4 Hours
10	22 Pa.s	45°C ± 5°C	40 rpm	8 Hours

All air is eliminated from the solution before use by placing it in a vacuum for two hours (minimum 0.01 mbar). It is possible to sterilize a solution prepared with non-sterile material by filtering it through a filter with a pore size of 0.22 μ m. In this case, to facilitate filtration, the solution can first be prepared at a lower viscosity than will be required for its application. It is then filtered and subsequently distilled under vacuum until it reaches the concentration corresponding to the desired viscosity. Solutions thus prepared can be mixed with bone granules to form a paste to be used to fill bone cavities and defects. The ratio between the quantity of solution and the quantity of granules is 1:3, w/w, or more. If the quantity of solution is too small, the granules will not be sufficiently bound together, so more solution must be added. If the paste is too liquid for easy application, it can be placed in a high vacuum for about two minutes, repeating this operation until the correct consistency is obtained.

The bone granules that can be used in the present invention are not particularly limited. In general, it is possible to use those already in common use. Examples of these can be found in U.S. Patents Nos. 4,693,986 and 4,629,464. The diameter of the granules can be between 50 μ m and 5mm, and they can be porous or nonporous. Among

the materials preferred due to their biocompatibility are granules of hydroxyapatite, tricalcium phosphate, and calcium carbonate.

For purely illustrative purposes, described hereafter are some examples of preparations of solutions and pastes according to the present invention.

The Esters of Hyaluronic Acid

Esters of hyaluronic acid useful in the present invention are esters of hyaluronic acid with aliphatic, araliphatic, cycloaliphatic or heterocyclic alcohols, in which are esterified all (so-called "total esters") or only a part (so-called "partial esters") of the carboxylic groups of the hyaluronic acid, and salts of the partial esters with metals or with organic bases, biocompatible or acceptable from a pharmacological point of view.

The useful esters include esters which derive from alcohols which themselves possess a notable pharmacological action. The saturated alcohols of the aliphatic series or simple alcohols of the cycloaliphatic series are useful in the present invention.

In the above mentioned esters in which some of the carboxylic acid groups remain free (i.e., partial esters), these may be salified with metals or organic bass, such as with alkaline or alkaline earth metals or with ammonia or nitrogenous organic bases.

Most of the esters of hyaluronic acid ("HY"), unlike HY itself, present a certain degree of solubility in organic solvents. This solubility depends on the percentage of esterified carboxylic groups and on the type of alkyl group linked with the carboxyl. Therefore, an HY compound with all its carboxylic groups esterified presents, at room temperature, good

solubility for example in dimethylsulfoxide (the benzyl ester of HY dissolves in DMSO in a measure of 200 mg/ml). Most of the total esters of HY present also, unlike HY and especially its salts, poor solubility in water and are essentially insoluble in water. The solubility characteristics, together with particular and notable viscoelastic properties, make the HY esters particularly preferred for use in composite membranes.

Alcohols of the aliphatic series to be used as esterifying components of the carboxylic groups of hyaluronic acid for use in composite membranes according to the present invention are for example those with a maximum of 34 carbon atoms, which may be saturated or unsaturated and which may possibly also be substituted by other free functional or functionally modified groups, such as amine, hydroxyl, aldehyde, ketone, mercaptan, or carboxyl groups or by groups derived from these, such as hydrocarbyl or di-hydrocarbylamine groups (from now on the term "hydrocarbyl" will be used to refer not only to monovalent radicals of hydrocarbons such as the C_nH_{2n+1} type, but also bivalent or trivalent radicals, such as "alkylenes" C_nH_{2n} or "alkylidenes" C_nH_{2n}), ether or ester groups, acetal or ketal groups, thioether or thioester groups, and esterified carboxyl or carbamide groups and carbamide substituted by one or more hydrocarbyl groups, by nitrile groups or by halogens.

Of the above mentioned groups containing hydrocarbyl radicals, these are preferably lower aliphatic radicals, such as alkyls, with a maximum of 6 carbon atoms. Such alcohols may also be interrupted in the carbon atom chain by heteroatoms, such as oxygen, nitrogen and sulfur atoms. Preferred are alcohols substituted with one or two of the said functional groups.

Alcohols of the above mentioned group which are preferably used are those with a maximum of 12, and especially 6 carbon atoms, and in which the hydrocarbyl atoms in the above mentioned amine, ether, ester, thioether, thioester, acetal, ketal groups represent alkyl groups with a maximum of 4 carbon atoms, and also in the esterified carboxyl or substituted carbamide groups the hydrocarbyl groups are alkyls with the same number of carbon atoms, and in which in the amine or carbamide groups may be alkylenamine or alkylencarbamide groups with a maximum of 8 carbon atoms. Of these alcohols, specifically preferred are saturated and non-substituted alcohols, such as the methyl, ethyl, propyl, and isopropyl alcohols, normal butyl alcohol, isobutyl alcohol, tertiary butyl alcohol, the amyl, pentyl, hexyl, octyl, nonyl and dodecyl alcohols and, above all, those with a linear chain, such as normal octyl and dodecyl alcohols. Of the substituted alcohols of this group, the bivalent alcohols are useful, such as ethyleneglycol, propyleneglycol and butyleneglycol, the trivalent alcohols such as glycerine, the aldehyde alcohols such as tartronic alcohol, the carboxylic alcohols such as lactic acids, for example glycolic acid, malic acid, the tartaric acids, citric acid, the aminoalcohols, such as normal aminoethanol, aminopropanol, normal aminobutanol and their dimethylated and diethylated derivatives in the amine function, choline, pyrrolidinyethanol, piperidinyethanol, piperazineylethanol and the corresponding derivatives of normal propyl or normal butyl alcohol, monothioethyleneglycol or its alkyl derivatives, such as the ethyl derivative in the mercaptan function.

Of the higher saturated aliphatic alcohols, preferred are cetyl alcohol and myricyl alcohol, but for the aim of the present invention the higher unsaturated alcohols with one or two double bonds, are especially important, such as especially those contained in many essential oils and with affinity to terpene, such as citronellol, geraniol, nerol, nerolidol, linalool, farnesol, phytol. of the unsaturated lower alcohols it is necessary to consider allyl alcohol and propargyl alcohol. Of the araliphatic alcohols, preferred are those with only one benzene residue and in which the aliphatic chain has a maximum of 4 carbon atoms, which the benzene residue can be substituted by between 1 and 3 methyl or hydroxyl groups or by halogen atoms, especially by chlorine, bromine and iodine, and in which the aliphatic chain may be substituted by one or more functions chosen from the group containing free amine groups or mono- or dimethylated or by pyrrolidine or piperidine groups. Of these alcohols, most preferred are benzyl alcohol and phenetyl alcohol.

The alcohols of the cycloaliphatic or aliphatic-cycloaliphatic series may derive from mono- or polycyclic hydrocarbons, may preferably have a maximum of 34 carbon atoms, may be unsubstituted and may contain one or more substituents, such as those mentioned above for the aliphatic alcohols. Of the alcohols derived from cyclic monoannular hydrocarbons, preferred are those with a maximum of 12 carbon atoms, the rings with preferably between 5 and 7 carbon atoms, which may be substituted for example by between one and three lower alkyl groups, such as methyl, ethyl, propyl or isopropyl groups. As specific alcohols of this group the following are most preferred: cyclohexanol, cyclohexanediol, 1,2,3-cyclohexanetriol and 1,3,5-cyclohexanetriol (phloroglucitol), inositol, and the

alcohols which derive from p-methane such as carvomenthol, menthol, and α - γ -terpineol, 1-terpineol, 4-terpineol and piperitol, or the mixture of these alcohols known as "terpineol", 1,4- and 1,8 terpin. Of the alcohols which derive from hydrocarbons with condensed rings, such as those of the thujane, pinane or comphane, the following are preferred: thujanol, sabinol, pinol hydrate, D and L-borneol and D and L-isoborneol.

Aliphatic-cycloaliphatic polycyclic alcohols to be used for the esters of the present invention are sterols, cholic acids and steroids, such as sexual hormones and their synthetic analogues, especially corticosteroids and their derivatives. It is therefore possible to use: cholesterol, dihydrocholesterol, epidihydrocholesterol, coprostanol, epicoprostanol, sitosterol, stigmasterol, ergosterol, cholic acid, deoxycholic acid, lithocholic acid, estriol, estradiol, equilenin, equilin and their alkylate derivatives, as well as their ethynyl or propynyl derivatives in position 17, such as 17 α -ethynyl-estradiol or 7 α -methyl-17 α -ethynyl-estradiol, pregnenolone, pregnanediol, testosterone and its derivatives, such as 17 α -methyltestosterone, 1,2-dehydrotestosterone and 17 α -methyl-1,2-dehydrotestosterone, the alkynylate derivatives in position 17 of testosterone and 1,2-dehydrotestosterone, such as 17 α -ethynyltestosterone, 17 α -propynyltestosterone, norgestrel, hydroxyprogesterone, corticosterone, deoxycorticosterone, 19-nortestosterone, 19-nor-17 α -methyltestosterone and 19-nor-17 α -ethynyltestosterone, antihormones such as cyproterone, cortisone, hydrocortisone, prednisone, prednisolone, fluorocortisone, dexamethasone, betamethasone, paramethasone, flumethasone, fluocinolone,

fluprednylidene, clobetasol, beclomethasone, aldosterone, deoxycorticosterone, alfaxolone, alfadolone, and bolasterone. As esterifying components for the esters of the present invention the following
5 are useful: genins (aglycons) of the cardioactive glucosides, such as digitoxigenin, gitoxigenin, digoxigenin, strophanthidin, tigogenin and saponins.

Other alcohols to be used according to the invention are the vitamin ones, such as axerophthol,
10 vitamins D₂ and D₃, aneurine, lactoflavine, ascorbic acid, riboflavine, thiamine, and pantothenic acid.

Of the heterocyclic acids, the following can be considered as derivatives of the above mentioned cycloaliphatic or aliphatic-cycloaliphatic alcohols if
15 their linear or cyclic chains are interrupted by one or more, for example by between one and three heteroatoms, for instance chosen from the group formed by -O-, -S-, -N, and -NH-, and in these, there may be one or more unsaturated bonds, for example double bonds, in
20 particular between one and three, thus including also heterocyclic compounds with aromatic structures. For example the following should be mentioned: furfuryl alcohol, alkaloids and derivatives such as atropine, scopolamine, cinchonine, la cinchonidine, quinine,
25 morphine, codeine, nalorphine, N-butylscopolammonium bromide, ajmaline; phenylethylamines such as ephedrine, isoproterenol, epinephrine; phenothiazine drugs such as perphenazine, pipothiazine, carphenazine, homofenazine, acetophenazine, fluophenazine, and N-
30 hydroxyethylpromethazine chloride; thioxanthene drugs such as flupenthixol and clopenthixol; anticonvulsants such as meprophenidol; antipsychotics such as opipramol; antiemetics such as oxypendyl; analgesics such as carbetidine and phenoperidine and methadol; hypnotics
35 such as etodroxizine; anorexics such as benzydrol and

diphemethoxidine; minor tranquilizers such as hydroxyzine; muscle relaxants such as cinnamedrine, diphylline, mephenesin, methocarbamol, chlorphenesin, 2,2-diethyl-1,3-propanediol, guaifenesin, hydrocylamide; 5 coronary vasodilators such as dipyridamole and oxyfedrine; adrenergic blockers such as propanolol, timolol, pindolol, bupranolol, atenolol, metoprolol, practolol; antineoplastics such as 6-azauridine, cytarabine, floxuridine; antibiotics such as 10 chloramphenicol, thiamphenicol, erythromycin, oleandomycin, lincomycin; antivirals such as idoxuridine; peripheral vasodilators such as isonicotinyl alcohol; carbonic anhydrase inhibitors such as sulocarbilate; antiasthmatic and antiinflammatories 15 such as tiaramide; sulfamidics such as 2-p-sulfanilonoethanol.

In some cases hyaluronic acid esters may be of interest where the ester groups derive from two or more therapeutically active hydroxylic substances, and 20 naturally all possible variants may be obtained. Especially interesting are the substances in which two types of different ester groups deriving from drugs of a hydroxylic character are present and in which the remaining carboxyl groups are free, salified with metals 25 or with a base, possibly also the bases being themselves therapeutically active, for example with the same or similar activity as that of the esterifying component. In particular, it is possible to have hyaluronic esters deriving on the one hand from an antiinflammatory 30 steroid, such as one of those mentioned previously, and on the other hand from a vitamin, from an alkaloid or from an antibiotic, such as one of those listed.

Method of Preparing HY Esters of the InventionMethod A:

The esters of hyaluronic acid may be prepared by methods known per se for the esterification of carboxylic acids, for example by treatment of free hyaluronic acid with the desired alcohols in the presence of catalyzing substances, such as strong inorganic acids or ionic exchangers of the acid type, or with an etherifying agent capable of introducing the desired alcoholic residue in the presence of inorganic or organic bases. As esterifying agents it is possible to use those known in literature, such as especially the esters of various inorganic acids or of organic sulphonic acids, such as hydracids, that is hydrocarbyl halogenides, such as methyl or ethyl iodide, or neutral sulphates or hydrocarbyl acids, alfites, carbonates, silicates, phosphites or hydrocarbyl sulfonates, such as methyl benzene or p-toluene-sulfonate or methyl or ethyl chlorosulfonate. The reaction may take place in a suitable solvent, for example an alcohol, preferably that corresponding to the alkyl group to be introduced in the carboxyl group. But the reaction may also take place in non-polar solvents, such as ketones, ethers, such as dioxane or aprotic solvents, such as dimethylsulphoxide. As a base it is possible to use for example a hydrate of an alkaline or alkaline earth metal or magnesium or silver oxide or a basic salt or one of these metals, such as a carbonate, and, of the organic bases, a tertiary azotized base, such as pyridine or collidine. In the place of the base it is also possible to use an ionic exchanger of the basic type.

Another esterification method employs the metal salts or salts with organic azotized bases, for example ammonium or ammonium substitute salts. Preferably, the salts of the alkaline or alkaline earth metals are used,

but also any other metallic salt may be used. The esterifying agents are also in this case those mentioned above and the same applies to the solvents. It is preferable to use aprotic solvents, for example
5 dimethylsulphoxide and dimethylformamide.

In the esters obtained according to this procedure or according to the other procedure described hereafter, free carboxylic groups of the partial esters may be salified, if desired, in a per se known manner.

10

Method B:

The hyaluronic esters may also be prepared by a method which consists of treating a quaternary ammonium salt of hyaluronic acid with an etherifying agent,
15 preferably in an aprotic organic solvent.

As organic solvents it is preferable to use aprotic solvents, such as dialkylsulphoxides, dialkylcarboxamides, such as in particular lower alkyl
20 dialkylsulphoxides, especially dimethyl-sulphoxide, and lower alkyl dialkylamides of lower aliphatic acids, such as dimethyl or diethyl-formamide or dimethyl or diethylacetamide.

Other solvents however are to be considered which are not always aprotic, such as alcohols, ethers,
25 ketones, esters, especially aliphatic or heterocyclic alcohols and ketones with a lower boiling point, such as hexafluoroisopropanol, trifluoroethanol, and N-methylpyrrolidone.

The reaction is effected preferably at a
30 temperature range of between about 0°C and 100°C, especially between about 25°C and 75°C, for example at about 30°C.

The esterification is carried out preferably by adding by degrees the esterifying agent to the above mentioned ammonium salt to one of the above mentioned solvents, for example to dimethyl-sulphoxide.

5 As an alkylating agent it is possible to use those mentioned above, especially the hydrocarbonyl halogens, for example alkyl halogens. As starting quaternary ammonium salts it is preferable to use the lower ammonium tetraalkylates, with alkyl groups preferably
10 between 1 and 6 carbon atoms. Mostly, hyaluronate of tetrabutylammonium is used. It is possible to prepare these quaternary ammonium salts by reacting a metallic salt of hyaluronic acid, preferably one of those mentioned above, especially sodium or potassium salt, in
15 aqueous solution with a salified sulphonic resin with a quaternary ammonium base.

One variation of the previously described procedure consists in reacting a potassium or sodium salt of hyaluronic acid, suspended in a suitable solution such
20 as dimethylsulphoxide, with a suitable alkylating agent in the presence of catalytic quantities of a quaternary ammonium salt, such as iodide of tetrabutylammonium.

For the preparation of the hyaluronic acid esters, it is possible to use hyaluronic acids of any origin,
25 such as for example the acids extracted from the above mentioned natural starting materials, for example from cocks' combs. The preparation of such acids is described in literature: preferably, purified hyaluronic acids are used. Especially used are hyaluronic acids
30 comprising molecular fractions of the integral acids obtained directly by extraction of the organic materials with molecular weights varying within a wide range, for example from about 90%-80% (MW = 11.7 - 10.4 million) to
35 0.2% (MW = 30,000) of the molecular weight of the integral acid having a molecular weight of 13 million,

preferably between 5% and 0.2%. Such fractions may be obtained with various procedures described in literature, such as by hydrolyzing, oxydizing, enzymatic or physical procedures, such as mechanical or radiational procedures. Primordial extracts are therefore often formed during these same by publication procedures (for example see the article by Balazs et al. quoted above in "Cosmetics & Toiletries"). The separation and purification of the molecular fractions obtained are brought about by known techniques, for example by molecular filtration.

Additionally useful are purified fractions obtainable from hyaluronic acid, such as for example the ones described in European Patent Publn. No. 0138572.

The salification of HY with the above metals, for the preparation of starting salts for the particular esterification procedure described above, is performed in a per se known manner, for example by reacting HY with the calculated base quantity, for example with alkaline hydrates or with basic salts of such metals, such as carbonates or bicarbonates.

In the partial esters it is possible to salify all the remaining carboxylic groups or only part of them, dosing the base quantities so as to obtain the desired stoichiometric degree of salification. With the correct degree of salification it is possible to obtain esters with a wide range of different dissociation constants and which therefore give the desired pH, in solution or "in situ" at the time of therapeutic application.

Preparation Examples:

The following exemplify the preparation of hyaluronic acid esters useful in the composite membranes of the present invention.

Example 1 - Preparation of the (partial) propyl ester of hyaluronic acid (HY).

- 50% of the esterified carboxylic groups

5 - 50% of the salified carboxylic groups (Na)

12.4 g of HY tetrabutylammonium salt with a molecular weight 170,000 corresponding to 20 m.Eq. of a monomeric unit are solubilized in 620 ml of dimethylsulfoxide at 25°C, 1.8 g (10.6 m.Eq.) of propyl iodide are added and the resulting solution is kept at a temperature of 30° for 12 hours.

A solution containing 62 ml of water and 9 g of sodium chloride is added and the resulting mixture is slowly poured into 3,500 ml of acetone under constant agitation. A precipitate is formed which is filtered and washed three times with 500 ml of acetone/water 5:1 and three times with acetone and finally vacuum dried for eight hours at 30°C.

The product is then dissolved in 550 ml of water containing 1% of sodium chloride and the solution is slowly poured into 3,000 ml of acetone under constant agitation. A precipitate is formed which is filtered and washed twice with 500 ml of acetone/water (5:1) and three times with 500 ml of acetone and finally vacuum dried for 24 hours at 30°C. 7.9 g of the partial propyl ester compound in the title are obtained. Quantitative determination of the ester groups is carried out using the method of R.H. Cundiff and P.C. Markunas [Anal. Chem. 33, 1028-1030, (1961)].

30

Example 2 - Preparation of the (partial) isopropyl ester of hyaluronic acid (HY) - 50% of esterified carboxylic groups - 50% of salified carboxylic groups (Na)

35 12.4 g of HY tetrabutylammonium salt with a molecular weight of 160,000 corresponding to 20 m.Eq. of a monomeric unit are solubilized in 620 ml of

dimethylsulfoxide at 25°C, 1.8 g (10.6 m.Eq.) of isopropyl iodide are added and the resulting solution is kept for 12 hours at 30°C.

5 A solution containing 62 ml of water and 9 g of sodium chloride is added and the resulting mixture is slowly poured into 3,500 ml of acetone under constant agitation. A precipitate is formed which is filtered and washed three times with 500 ml of acetone/water 5:1 and three times with acetone and finally vacuum dried
10 for eight hours at 30°C.

The product is then dissolved in 550 ml of water containing 1% of sodium chloride and the solution is slowly poured into 3,000 ml of acetone under constant agitation. A precipitate is formed which is filtered
15 and washed twice with 500 ml of acetone/water 5:1 and three times with 500 ml of acetone and finally vacuum dried for 24 hours at 30°C. 7.8 g of the partial isopropyl ester compound in the title are obtained. Quantitative determination of the ester groups is
20 carried out using the method of R.H. Cundiff and P.C. Markunas [Anal. Chem. 33, 1028-1030 (1961)].

25 Example 3 - Preparation of the (partial) ethyl ester of hyaluronic acid (HY) - 75% of esterified carboxylic groups - 25% of salified carboxylic groups (Na)

12.4 g of HY tetrabutylammonium salt with a molecular weight of 250,000 corresponding to 20 m.Eq. of a monomeric unit are solubilized in 620 ml of
30 dimethylsulfoxide at 25°C, 2.5 g (15.9 m.Eq.) of ethyl iodide are added and the resulting solution is kept for 12 hours at 30°C.

A solution containing 62 ml of water and 9 g of sodium chloride is added and the resulting mixture is
35 slowly poured into 3,500 ml of acetone under constant agitation. A precipitate is formed which is filtered

and washed three times with 500 ml of acetone/water 5:1 and three times with acetone and finally vacuum dried for eight hours at 30°C.

The product is then dissolved in 550 ml of water containing 1% of sodium chloride and the solution is slowly poured into 3,000 ml of acetone under constant agitation. A precipitate is formed which is filtered and washed twice with 500 ml of acetone/water 5:1 and three times with 500 ml of acetone and finally vacuum dried for 24 hours at 30°C. 7.9 g of the partial ethyl ester compound in the title are obtained. Quantitative determination of the ester groups is carried out using the method of R.H. Cundiff and P.C. Markunas [Anal. Chem. 33, 1028-1030, (1961)].

Example 4 - Preparation of the (partial) methyl ester of hyaluronic acid (HY) - 75% of esterified carboxylic groups - 25% of salified carboxylic groups (Na)

12.4 g of HY tetrabutylammonium salt with a molecular weight of 80,000 corresponding to 20 m.Eq. of a monomeric unit are solubilized in 620 ml of dimethylsulfoxide at 25°C, 2.26 g (15.9 m.Eq.) of methyl iodide are added and the resulting solution is kept for 12 hours at 30°C.

A solution containing 62 ml of water and 9 g of sodium chloride is added and the resulting mixture is slowly poured into 3,500 ml of acetone under constant agitation. A precipitate is formed which is filtered and washed three times with 500 ml of acetone/water 5:1 and three times with acetone and finally vacuum dried for eight hours at 30°C.

The product is then dissolved in 550 ml of water containing 1% of sodium chloride and the solution is slowly poured into 3,000 ml of acetone under constant agitation. A precipitate is formed which is filtered

and washed twice with 500 ml of acetone/water 5:1 and three times with 500 ml of acetone and finally vacuum dried for 24 hours at 30°C. 7.8 g of the partial methyl ester compound in the title are obtained. Quantitative
5 determination of the ester groups is carried out using the method of R.H. Cundiff and P.C. Markunas [Anal. Chem. 33, 1028-1030 (1961)].

10 Example 5 - Preparation of the methyl ester of hyaluronic acid (HY)

12.4 g of HY tetrabutylammonium salt with a molecular weight of 120,000 corresponding to 20 m.Eq. of a monomeric unit are solubilized in 620 ml of
15 dimethylsulfoxide at 25°C, 3 g (21.2 m.Eq.) of methyl iodide are added and the solution is kept for 12 hours at 30°C.

The resulting mixture is slowly poured into 3,500 ml of ethyl acetate under constant agitation. A
20 precipitate is formed which is filtered and washed four times with 500 ml of ethyl acetate and finally vacuum dried for twenty four hours at 30°C.

8 g of the ethyl ester product in the title are obtained. Quantitative determination of the ester
25 groups is carried out using the method of R.H. Cundiff and P.C. Markunas [Anal. Chem. 33, 1028-1030 (1961)].

30 Example 6 - Preparation of the ethyl ester of hyaluronic acid (HY)

12.4 g of HY tetrabutylammonium salt with a molecular weight of 85,000 corresponding to 20 m.Eq. of a monomeric unit are solubilized in 620 ml of
35 dimethylsulfoxide at 25°C, 3.3 g (21.2 m.Eq.) of ethyl iodide are added and the solution is kept for 12 hours at 30°C.

The resulting mixture is slowly poured into 3,500 ml of ethyl acetate under constant agitation. A precipitate is formed which is filtered and washed four times with 500 ml of ethyl acetate and finally vacuum dried for twenty-four hours at 30°C.

8 g of the ethyl ester product in the title are obtained. Quantitative determination of the ester groups is carried out using the method of R.H. Cundiff and P.C. Markunas [Anal. Chem. 33, 1028-1030 (1961)].

10

Example 7 - Preparation of the propyl ester of hyaluronic acid (HY)

12.4 g of HY tetrabutylammonium salt with a molecular weight of 170,000 corresponding to 20 m.Eq. of a monomeric unit are solubilized in 620 ml of dimethylsulfoxide at 25°C, 3.6 g (21.2 m.Eq.) of propyl iodide are added and the solution is kept for 12 hours at 30°C.

The resulting mixture is slowly poured into 3,500 ml of ethyl acetate under constant agitation. A precipitate is formed which is filtered and washed four times with 500 ml of ethyl acetate and finally vacuum dried for twenty-four hours at 30°C.

8.3 g of the propyl ester product in the title are obtained. Quantitative determination of the ester groups is carried out using the method of R.H. Cundiff and P.C. Markunas [Anal. Chem. 33, 1028-1030 (1961)].

Example 8 - Preparation of the (partial) butyl ester of hyaluronic acid (HY) - 50% of esterified carboxylic groups - 50% of salified carboxylic groups (Na)

12.4 g of HY tetrabutylammonium salt with a molecular weight of 620,000 corresponding to 20 m.Eq. of a monomeric unit are solubilized in 620 ml of dimethylsulfoxide at 25°C, 1.95 g (10.6 m.Eq.) of

n-butyl iodide are added and the resulting solution is kept for 12 hours at 30°C.

5 A solution containing 62 ml of water and 9 g of sodium chloride is added and the resulting mixture is slowly poured into 3,500 ml of acetone under constant agitation. A precipitate is formed which is filtered and washed three times with 500 ml of acetone/water 5:1 and three times with acetone and finally vacuum dried for eight hours at 30°C.

10 The product is then dissolved in 550 ml of water containing 1% of sodium chloride and the solution is slowly poured into 3,000 ml of acetone under constant agitation. A precipitate is formed which is filtered and washed twice with 500 ml of acetone/water 5:1 and
15 three times with 500 ml of acetone and finally vacuum dried for 24 hours at 30°C. 8 g of the partial butyl ester compound in the title are obtained. Quantitative determination of the ester groups is carried out using the method of R.H. Cundiff and P.C. Markunas [Anal.
20 Chem. 33, 1028-1030 (1961)].

25 Example 9 - Preparation of the (partial) ethoxy-carbonylmethyl ester of hyaluronic acid (HY) - 75% of esterified carboxylic groups - 25% of salified carboxylic groups (Na)

30 12.4 g of HY tetrabutylammonium salt with a molecular weight of 180,000 corresponding to 20 m.Eq. of a monomeric unit are solubilized in 620 ml of dimethylsulfoxide at 25°C, 2 g of tetrabutylammonium iodide and 1.84 g (15 m.Eq.) of ethyl chloroacetate are added and the resulting solution of kept for 24 hours at 30°C.

A solution containing 62 ml of water and 9 g of sodium chloride is added and the resulting mixture is slowly poured into 3,500 ml of acetone under constant agitation. A precipitate is formed which is filtered and washed three times with 500 ml of acetone/water 5:1 and three times with acetone and finally vacuum dried for eight hours at 30°C.

The product is then dissolved in 550 ml of water containing 1% of sodium chloride and the solution is slowly poured into 3,000 ml of acetone under constant agitation. A precipitate is formed which is filtered and washed twice with 500 ml of acetone/water 5:1 and three times with 500 ml of acetone and finally vacuum dried for 24 hours at 30°C. 10 g of the partial ethoxycarbonyl methyl ester compound in the title are obtained.

Quantitative determination of the ethoxylic ester groups is carried out using the method of R.H. Cundiff and P.C. Markunas [Anal. Chem. 33, 1028-1030 (1961)].

Example 10 - Preparation of the n-pentyl ester of hyaluronic acid (HY)

12.4 g of HY tetrabutylammonium salt with a molecular weight of 620,000 corresponding to 20 m.Eq. of a monomeric unit are solubilized in 620 ml of dimethylsulfoxide at 25°C, 3.8 g (25 m.Eq.) of n-pentyl bromide and 0.2 g of iodide tetrabutyl-ammonium are added, the solution is kept for 12 hours at 30°C.

The resulting mixture is slowly poured into 3,500 ml of ethyl acetate under constant agitation. A precipitate is formed which is filtered and washed four times with 500 ml of ethyl acetate and finally vacuum dried for twenty four hours at 30°C.

8.7 g of the n-pentyl ester product in the title are obtained. Quantitative determination of the ester groups is carried out using the method described on pages 169-172 of Siggia S. and Hann J.G. "Quantitative organic analysis via functional groups" 4th Edition, John Wiley and Sons.

Example 11 - Preparation of the isopentyl ester of hyaluronic acid (HY)

10

12.4 g of HY tetrabutylammonium salt with a molecular weight of 170,000 corresponding to 20 m.Eq. of a monomeric unit are solubilized in 620 ml of dimethylsulfoxide at 25°C, 3.8 g (25 m.Eq.) of isopentyl bromide and 0.2 g of tetrabutylammonium iodide are added, the solution is kept for 12 hours at 30°C.

The resulting mixture is slowly poured into 3,500 ml of ethyl acetate under constant agitation. A precipitate is formed which is filtered and washed four times with 500 ml of ethyl acetate and finally vacuum dried for twenty four hours at 30°C.

8.6 g of the isopentyl ester product featured in the title are obtained. Quantitative determination of the ester groups is carried out according to the method described on pages 169-172 of Siggia S. and Hanna J.G. "Quantitative organic analysis via functional groups" 4th Edition, John Wiley and Sons.

Example 12 - Preparation of the benzylester of hyaluronic acid (HY)

30

12.4 g of HY tetrabutylammonium salt with a molecular weight of 170,000 corresponding to 20 m.Eq. of a monomeric unit are solubilized in 620 ml of dimethylsulfoxide at 25°C, 4.5 g (25 m.Eq.) of benzyl bromide and 0.2 g of tetrabutylammonium iodide are added, the solution is kept for 12 hours at 30°C.

The resulting mixture is slowly poured into 3,500 ml of ethyl acetate under constant agitation. A precipitate is formed which is filtered and washed four times with 500 ml of ethyl acetate and finally vacuum
5 dried for twenty four hours at 30°C.

9 g of the benzyl ester product in the title are obtained. Quantitative determination of the ester groups is carried out according to the method described on pages 169-172 of Siggia S. and Hanna J.G.
10 "Quantitative organic analysis via functional groups" 4th Edition, John Wiley and Sons.

Example 13 - Preparation of the β -phenylethyl ester of hyaluronic acid (HY)

15 12.4 g of HY tetrabutylammonium salt with a molecular weight of 125,000 corresponding to 20 m.Eq. of a monomeric unit are solubilized in 620 ml of dimethylsulfoxide at 25°C, 4.6 g (25 m.Eq.) of
20 2-bromoethylbenzene and 185 mg of tetrabutylammonium iodide are added, and the solution is kept for 12 hours at 30°C.

The resulting mixture is slowly poured into 3,500 ml of ethyl acetate under constant agitation. A
25 precipitate is thus formed which is then filtered and washed four times with 500 ml of ethyl acetate and finally vacuum dried for twenty four hours at 30°C.

9.1 g of the β -phenylethyl ester in the title are obtained. Quantitative determination of the ester
30 groups is carried out according to the method described on page 168-172 of Siggia S. and hanna J.G. "Quantitative organic analysis via functional groups" 4th Edition, John Wiley and Sons.

Example 14 - Preparation of the benzyl ester of hyaluronic acid (HY)

3 g of the potassium salt of HY with a molecular weight of 162,000 are suspended in 200 ml of dimethylsulfoxide; 120 mg of tetrabutylammonium iodide and 2.4 g of benzyl bromide are added.

The suspension is kept in agitation for 48 hours at 30°C. The resulting mixture is slowly poured into 1,000 ml of ethyl acetate under constant agitation. A precipitate is formed which is filtered and washed four times with 150 ml of ethyl acetate and finally vacuum dried for twenty four hours at 30°C.

3.1 g of the benzyl ester product in the title are obtained. Quantitative determination of the ester groups is carried out according to the method described on pages 169-172 of Siggia S. and Hanna J.G. "Quantitative organic analysis via functional groups" 4th Edition, John Wiley and Sons.

20

Example 15 - Preparation of the (partial propyl) ester of hyaluronic acid (HY) - 85% of esterified carboxylic groups - 15% of salified carboxylic groups (Na)

12.4 g of HY tetrabutylammonium salt with a molecular weight of 165,1000 corresponding to 20 m.Eq. of a monomeric unit are solubilized in 620 ml of dimethylsulfoxide at 25°C, 2.9 g (17 m.Eq.) of propyl iodide are added and the resulting solution is kept for 12 hours at 30°C.

A solution is then added containing 62 ml of water and 9 g of sodium chloride and the resulting mixture is slowly poured into 3,500 ml of acetone under constant agitation. A precipitate is formed which is filtered and washed three times with 500 ml of acetone/water 5:1 and three times with acetone and finally vacuum dried for eight hours at 30°C.

The product is then dissolved in 550 ml of water containing 1.% of sodium chloride and the solution is slowly poured into 3,000 ml of acetone under constant agitation. A precipitate is formed which is filtered and washed twice with 500 ml of acetone/water 5:1 and three times with 500 ml of acetone and finally vacuum dried for 24 hours at 30°C. 8 g of the partial propyl ester compound in the title are obtained. Quantitative determination of the ester groups is carried out using the method of R.H. Cundiff and P.C. Markunas [Anal. Chem. 33, 1028-1030 (1961)].

Example 16 - Preparation of the n-octyl ester of hyaluronic acid (HY)

12.4 g of HY tetrabutylammonium salt with a molecular weight of 170,000 corresponding to 20 m.Eq. of a monomeric unit are solubilized in 620 ml of dimethylsulfoxide at 25°C, 4.1 g (21.2 m.Eq.) of 1-bromooctane are added and the solution is kept for 12 hours at 30°C.

The resulting mixture is slowly poured into 3,500 ml of ethyl acetate under constant agitation. A precipitate is formed which is filtered and washed four times with 500 ml of ethyl acetate and finally vacuum dried for 24 hours at 30°C. 9.3 g of the octyl ester product in the title are obtained. Quantitative determination of the ester groups is carried out using the method described in Siggia S. and Hanna J.G. "Quantitative organic analysis via functional groups", 4th Edition, John Wiley and Sons, pages 169-172.

Example 17 - Preparation of the isopropyl ester of hyaluronic acid (HY)

12.4 g of HY tetrabutylammonium salt with a
5 molecular weight of 170.000 corresponding to 20 m.Eq. of
a monomeric unit are solubilized in 620 ml of
dimethylsulfoxide at 25°C, 2.6 g (21.2 m.Eq.) of
isopropyl bromide are added and the solution is kept
for 12 hours at 30°C.

10 The resulting mixture is slowly poured into 3,500
ml of ethyl acetate under constant agitation. A
precipitate is formed which is filtered and washed four
times with 500 ml of ethyl acetate and finally vacuum
dried for 24 hours at 30°C. 8.3 g of the isopropyl
15 ester product in the title are obtained. Quantitative
determination of the ester groups is carried out using
the method of R.H. Cundiff and P.C. Markunas (Anal.
Chem. 33, 1028-1030, 1961).

20 Example 18 - Preparation of the 2,6-dichlorobenzyl ester of hyaluronic acid (HY)

12.4 g of HY tetrabutylammonium salt with a
molecular weight of 170.000 corresponding to 20 m.Eq. of
25 a monomeric unit are solubilized in 620 ml of
dimethylsulfoxide at 25°C, 5.08 g (21.2 m.Eq.) of
2,6-dichlorobenzyl bromide are added and the solution is
kept for 12 hours at 30°C.

The resulting mixture is slowly poured into 3,500
30 ml of ethyl acetate under constant agitation. A
precipitate is formed which is filtered and washed four
times with 500 ml of ethyl acetate and finally vacuum
dried for 24 hours at 30°C. 9.7 g of the
2,6-dichlorobenzyl ester product in the title are
35 obtained. Quantitative determination of the ester
groups is carried out using the method described in
Siggia S. and Hanna J.G. "Quantitative organic analysis

via functional groups", 4th Edition, John Wiley and Sons, pages 169-172.

5 Example 19 - Preparation of the 4-terbutylbenzyl ester of hyaluronic acid (HY)

12.4 g of HY tetrabutylammonium salt with a molecular weight of 170,000 corresponding to 20 m.Eq. of a monomeric unit are solubilized in 620 ml of
10 dimethylsulfoxide at 25°C, 4.81 g (21.2 m.Eq.) of 4-terbutylbenzyl bromide are added and the solution is kept for 12 hours at 30°C.

The resulting mixture is slowly poured into 3,500 ml of ethyl acetate under constant agitation. A
15 precipitate is formed which is filtered and washed four times with 500 ml of ethyl acetate and finally vacuum dried for 24 hours at 30°C. 9.8 g of the 4-terbutylbenzyl ester product in the title are obtained. Quantitative determination of the ester
20 groups is carried out using the method described in Siggia S. and Hanna J.G. "Quantitative organic analysis via functional groups", 4th Edition, John Wiley and Sons, pages 169-172.

25 Example 20 - Preparation of the Heptadecyl ester of hyaluronic acid (HY)

12.4 g of HY tetrabutylammonium salt with a molecular weight of 170,000 corresponding to 20 m.Eq. of
30 a monomeric unit are solubilized in 620 ml of dimethylsulfoxide at 25°C, 6.8 g (21.2 M.Eq.) of Heptadecyl bromide are added and the solution is kept for 12 hours at 30°C.

The resulting mixture is slowly poured into 3,500
35 ml of ethyl acetate under constant agitation. A precipitate is formed which is filtered and washed four times with 500 ml of ethyl acetate and finally vacuum

dried for 24 hours at 30°C. 11 g of the Heptadecyl ester product in the title are obtained. Quantitative determination of the ester groups is carried out using the method described in Siggia S. and Hanna J.G.

5 "Quantitative organic analysis via functional groups", 4th Edition, John Wiley and Sons, pages 169-172.

Example 21 - Preparation of the Octadecyl ester of hyaluronic acid (HY)

10 12.4 g of HY tetrabutylammonium salt with a molecular weight of 170,000 corresponding to 20 m.Eq. of a monomeric unit are solubilized in 620 ml of dimethylsulfoxide at 25°C, 7.1 g (21.2 m.Eq.) of

15 octadecyl bromide are added and the solution is kept for 12 hours at 30°C.

The resulting mixture is slowly poured into 3,500 ml of ethyl acetate under constant agitation. A precipitate is formed which is filtered and washed four

20 times with 500 ml of ethyl acetate and finally vacuum dried for 24 hours at 30°C. 11 g of the octadecyl ester product in the title are obtained. Quantitative determination of the ester groups is carried out using the method described in Siggia S. and Hanna J.G.

25 "Quantitative organic analysis via functional groups", 4th Edition, John Wiley and Sons, pages 169-172.

Example 22 - Preparation of the 3-phenylpropyl ester of hyaluronic acid (HY)

30 12.4 g of HY tetrabutylammonium salt with a molecular weight of 170,000 corresponding to 20 m.Eq. of a monomeric unit are solubilized in 620 ml of dimethylsulfoxide at 25°C, 4.22 g (21.2 m.Eq.) of

35 3-phenylpropyl bromide are added and the solution is kept for 12 hours at 30°C.

The resulting mixture is slowly poured into 3,500 ml of ethyl acetate under constant agitation. A precipitate is formed which is filtered and washed four times with 500 ml of ethyl acetate and finally vacuum
5 dried for 24 hours at 30°C. 9 g of the 3-phenylpropyl ester product in the title are obtained. Quantitative determination of the ester groups is carried out using the method described in Siggia S. and Hanna J.G. "Quantitative organic analysis via functional groups",
10 4th Edition, John Wiley and Sons, pages 169-172.

Example 23 - Preparation of the 3,4,5-trimethoxy-benzyl ester of hyaluronic acid (HY)

15 12.4 g of HY tetrabutylammonium salt with a molecular weight of 170,000 corresponding to 20 M.Eq. of a monomeric unit are solubilized in 620 ml of dimethylsulfoxide at 25°C, 4.6 g (21.2 m.Eq.) of 3,4,5-trimethoxybenzyl chloride are added and the
20 solution is kept for 12 hours at 30°C.

The resulting mixture is slowly poured into 3,500 ml of ethyl acetate under constant agitation. A precipitate is formed which is filtered and washed four times with 500 ml of ethyl acetate and finally vacuum
25 dried for 24 hours at 30°C. 10 g of the 3,4,5-trimethoxybenzyl ester product in the title are obtained. Quantitative determination of the ester groups is carried out using the method described in Siggia S. and Hanna J.G. "Quantitative organic analysis
30 via functional groups", 4th Edition, John Wiley and Sons, pages 169-172.

Example 24 - Preparation of the Cinnamyl ester of hyaluronic acid (HY)

12.4 g of Hy tetrabutylammonium salt with a
5 molecular weight of 170,000 corresponding to 20 m.Eq. of
a monomeric unit are solubilized in 620 ml of
dimethylsulfoxide at 25°C, 4.2 g (21.2 m.Eq.) of
Cinnamyl bromide are added and the solution is kept for
12 hours at 30°C.

10 The resulting mixture is slowly poured into 3,500
ml of ethyl acetate under constant agitation. A
precipitate is formed which is filtered and washed four
times with 500 ml of ethyl acetate and finally vacuum
dried for 24 hours at 30°C. 9.3 g of the Cinnamyl ester
15 product in the title are obtained. Quantitative
determination of the ester groups is carried out using
the method described in Siggia S. and Hanna J.G.
"Quantitative organic analysis via functional groups",
4th Edition, John Wiley and Sons, pages 169-172.

20

Example 25 - Preparation of the Decyl ester of hyaluronic acid (HY)

12.4 g of HY tetrabutylammonium salt with a
25 molecular weight of 170,000 corresponding to 20 m.Eq. of
a monomeric unit are solubilized in 620 ml of
dimethylsulfoxide at 25°C, 4.7 g (21.2 m.Eq.) of 1-bromo
decane are added and the solution is kept for 12 hours
at 30°C.

30 The resulting mixture is slowly poured into 3,500
ml of ethyl acetate under constant agitation. A
precipitate is formed which is filtered and washed four
times with 500 ml of ethyl acetate and finally vacuum
dried for 24 hours at 30°C. 9.5 g of the Decyl ester
35 product in the title are obtained. Quantitative
determination of the ester groups is carried out using
the method described in Siggia S. and Hanna J.G.

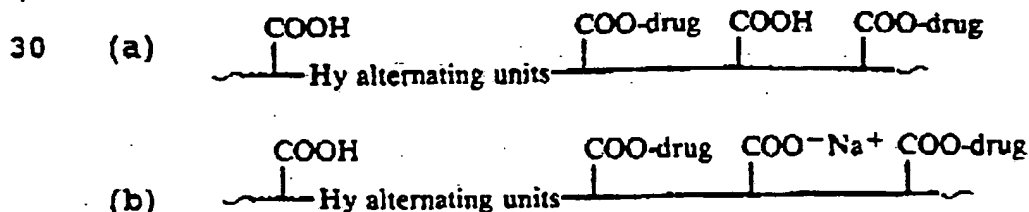
"Quantitative organic analysis via functional groups",
4th Edition, John Wiley and Sons, pages 169-172.

5 Example 26 - Preparation of the Nonyl ester of
hyaluronic acid (HY)

12.4 g of HY tetrabutylammonium salt with a
molecular weight of 170,000 corresponding to 20 m.Eq. of
a monomeric unit are solubilized in 620 ml of
10 dimethylsulfoxide at 25°C, 4.4 g (21.2 m.Eq.) of 1-bromo
nonane are added and the solution is kept for 12 hours
at 30°C.

The resulting mixture is slowly poured into 3,500
ml of ethyl acetate under constant agitation. A
15 precipitate is formed which is filtered and washed four
times with 500 ml of ethyl acetate and finally vacuum
dried for 24 hours at 30°C. 9 g of the Nonyl ester
product in the title are obtained. Quantitative
determination of the ester groups is carried out using
20 the method described in Siggia S. and Hanna J.G.
"Quantitative organic analysis via functional groups",
4th Edition, John Wiley and Sons, pages 169-172.

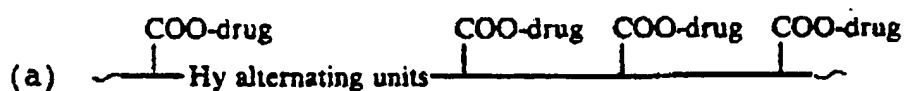
Complexes of hyaluronic acid and antibiotic drugs -
using partial salts of hyaluronic acid with a basic
25 active drug substance component, leaving the residue
acid groups of hyaluronic acid free or neutralizing them
with metals or bases. These complexes may be
represented as:





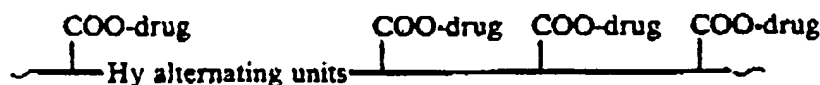
5

Complexes of hyaluronic acid and antibiotic drugs -
 using stoichiometrically neutral salts of HY with a
 basic drug substance component, adding further
 quantities of the drug component. These can be
 10 represented as:

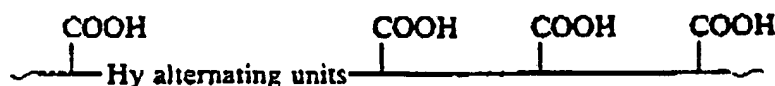


15

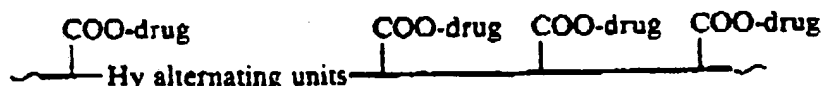
(b) Mixture of:



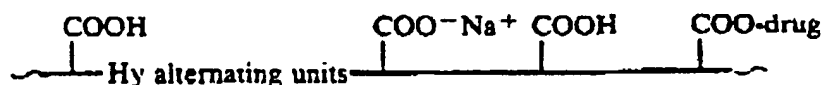
20



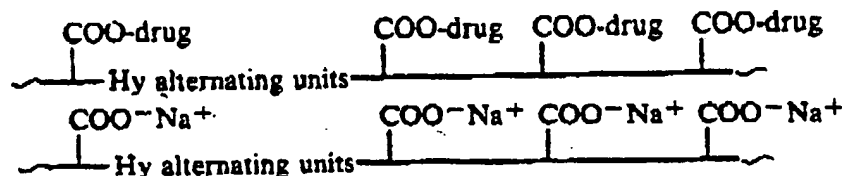
(c) Mixture of:



25



(d) Mixture of:

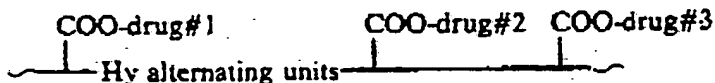


30

Complexes of hyaluronic acid and antibiotic drugs -
 using stoichiometrically neutral salts of HY with a
 basic drug substance component, adding further
 35 quantities of the drug component.

Complexes of hyaluronic acid and antibiotic drugs -
 using stoichiometrically neutral salts of HY with ad
 libitum mixtures of different drug components. These
 can be represented as:

5



Mixtures of any of the above possible medicaments
 10 can also be used in the present invention.

Examples of Active Drug Components

Drugs utilizable in the present invention can be
 various types, but are basic in nature, existing as a
 15 primary, secondary, tertiary, or quaternary amine,
 whereby the basic amine portion of the drug complexes
 with the acidic portion of the hyaluronic acid molecule.

Examples of pharmacologically active substances for
 use in medicaments according to the present invention
 20 are: basic and non basic antibiotics, for example
 aminoglucosidics, macrolides, tetracycline and peptides,
 such as for example gentamicin, neomycin, streptomycin,
 dihydrostreptomycin, kanamycin, amikacina, tobramycin,
 spectinomycin, erythromycin, oleandomycin, carbomycin,
 25 spiramycin, oxytetracycline, rolitetracycline,
 bacitracin, polymyxin B, gramicidin, colistin,
 chloramphenicol, lincomycin, vancomycin, novobiocin,
 ristocetin, clindamycin, amphotericin B, griseofulvin,
 nystatin and possibly their salts, such as sulphates or
 30 nitrates, or associations between the same or with other
 active principles, such as those mentioned hereafter.

Other drugs to be used to advantage according to
 the present invention are: other anti-infective agents
 such as diethylcarbamazine, mebendazole, the sulfamides
 35 such as sulfacetamide, sulfadiazine, sulfisoxazole;

antiviral and antitumoral agents such as iododeoxyuridine, adenine arabinoside, trifluorothymidine, aciclovir, ethyldeoxyuridine, bromovinyldeoxyuridine, and 5-iodo-5'-amino-2', 5'-dideoxyuridine.

Associations or mixtures of such drugs between themselves and possibly with other principles may also be used as antibiotic drugs according to the present invention. If in the place of only one active substance association or mixtures of active substances are used, such as these mentioned above, the salts of the basic active substances and hyaluronic acid and its molecular fractions may be mixed salts of one or more of such basic substances or possibly mixed salts of this type with a certain number of other acid groups of the polysaccharide salified with metals or bases.

Of the antibiotics, the following are of particular note: erythromycin, bacitracin, gentamicin, neomycin, aureomycin, gramicidin and their associations; of the antibacterials and disinfectants: nitrofurazone, mafenids, chlorhexidine, and derivatives of 8-hydroxyquinoline and possibly their salts. This list is of course only for illustrative purposes, and any other antibiotic agents known or described in literature may be used.

Method of Preparing Antibiotic Hyaluronic Acid Salts of the Present Invention

The preparation of the antibiotic salts according to the present invention may be carried out in a manner which is per se known, that is, by combining solutions or suspensions (in water or in organic solvents) of the components in calculated quantities and isolating the salts in an amorphous anhydrous form according to

per se known techniques. It is also possible to utilize bases or basic salts with alkaline or alkaline earth metals or magnesium or aluminum or ammonium. It is possible, for example, to (a) first prepare aqueous solutions of the two components, (b) freeze such components from aqueous solutions of their salts with acids of the respective metallic salts (for example sulphates and sodium salts) for treatment with ionic exchangers, and (c) unite the two solutions at a low temperature, for example between 0° and 20°. If the salt thus obtained is easily soluble in water, it should be freeze-dried, while salts which are not easily soluble may be separated by centrifugation, filtration or decantation, and possibly then dessicated.

15

Example 27 - Preparation of the Salt of Hyaluronic Acid (HY) with Streptomycin

2.43 g of streptomycin sulfate (10 mEq) are solubilized in 25 ml of distilled H₂O. The solution is eluted in a thermostatic column at 5°C, containing 15 ml of quaternary ammonium resin (Dowex 1x8) in OH⁻ form. The sulfate-free eluate is gathered in a thermostatic container at 5°C.

4.0 g of sodium salt of a hyaluronic acid with a molecular weight of 255,000 (corresponding to 10 mEq of a monomeric unit) are solubilized in 400 ml of distilled H₂O. The solution is then eluted in a thermostatic column at 5°C, containing 15 ml of sulfonic resin (Dowex 50x8) in H⁺ form. The sodium-free eluate is gathered under agitation in the solution of streptomycin base. The resulting solution is frozen and instantly freeze-dried. In the salt thus obtained, all the acidic groups of hyaluronic acid are salified with the basic functions of streptomycin. Yield: 5.5 g.

Microbiological determination on *B. subtilis* ATCC 6633 compared to standard streptomycin showed a content of 33.8% by weight of streptomycin base, corresponding to the theoretically calculated weight. Colorimetric determination of the glucuronic acid combined in polysaccharide according to the method of Bitter et al. (Anal. Biochem. 4, 330, 1962) shows a content in weight of HY acid of 66.2% (theoretical percentage - 66.0%).

10 Example 28 - Preparation of the Salt of a Hyaluronic Acid (HY) with Erythromycin

4.0 g of sodium salt of a hyaluronic acid with a molecular weight of 77,000 (corresponding to 10 mEq of a monomeric unit) are solubilized in 400 ml of distilled H₂O. The solution is then eluted in a thermostatic column at 5°C, containing 15 ml of sulfonic resin (Dowex 50x8) in H⁺ form. The eluate, free from sodium, is kept at a temperature of 5°C.

20 7.34 g of erythromycin base (10 mEq) are added to the solution of HY under agitation at 5°C, until complete solubilization is obtained. The resulting solution is frozen and freeze-dried. In the salt thus obtained all the acidic groups of hyaluronic acid are salified with erythromycin. Yield: 10.8 g.

30 Microbiological determination on *St. aureus* ATCC 6538 p in comparison with standard erythromycin shows a content of 66.0% by weight in erythromycin base, corresponding to the theoretical value. Colorimetric determination of the glucuronic acid combined in the polysaccharide according to the method of Bitter et al. (Anal. Biochem. 4, 330, 1962) shows a content of HY acid of 34.0%, corresponding to the theoretically calculated percentage.

Example 29 - Preparation of the Salt of a Hyaluronic Acid (HY) with Kanamycin

1.46 g of kanamycin sulphate (10 mEq) are
5 solubilized in 25 ml of distilled H₂O. The solution is
eluted in a thermostatic column at 5°C, containing 15 ml
of quaternary ammonium resin (Dowex 1x8) in OH⁻ form.
The eluate, free from sulfates is gathered in a
thermostatic container at 5°C.

10 4.0 g of the sodium salt of HY with a molecular
weight of 165,000 (corresponding to 10 mEq of a
monomeric unit) are solubilized in 400 ml of distilled
H₂O. The solution is then eluted in a thermostatic
15 column at 5°C, containing 15 ml of sulfonic resin (Dowex
50x8) in H⁺ form. The eluate, free from sodium, is
gathered under vortex agitation in the solution of
kanamycin base. The solution thus obtained is instantly
frozen and freeze-dried. Yield: 4.8 g. In the salt
obtained all the acid groups of HY are salified with
20 kanamycin.

Microbiological determination on *B. subtilis* ATCC
6633 in comparison with standard kanamycin shows a
content of 24.2% by weight of kanamycin base,
corresponding to the theoretically calculated
25 percentage. Colorimetric determination of the
glucuronic acid combined in polysaccharide according to
the method of Bitter et al. (Anal. Biochem. 4, 330,
1962) shows a content of HY acid of 75.8% in weight,
also corresponding to the theoretical content.

Example 30 - Preparation of the Salt of a Hyaluronic Acid (HY) with Neomycin

1.52 g of neomycin sulfate (10 mEq) are solubilized in 20 ml of distilled H₂O and eluted in a thermostatic column at 5°C, containing 15 ml of quaternary ammonium resin (Dowex 1x8) in OH⁻ form. The eluate, free from sulfates is gathered in a thermostatic container at 5°C.

4.0 g of HY sodium salt with a molecular weight of 170,000 (corresponding to 10 mEq of a monomeric unit) are solubilized in 400 ml of distilled H₂O and eluted in a thermostatic column at 5°C, containing 15 ml of sulfonic resin (Dowex 50x8) in H⁺ form. The eluate, free from sodium, is gathered under agitation in the solution of neomycin base. The viscoelastic precipitate which forms is separated by decantation and freeze-dried. Yield: 4.76 g. In the salt, all the HY acid groups are salified with neomycin.

Quantitative microbiological determination carried out on *St. aureus* ATCC 6538p compared to standard neomycin shows a content in weight of 21.2% of neomycin base, corresponding to the theoretically calculated value. Colorimetric determination of the glucuronic acid combined in polysaccharide according to the method of Bitter et al. (Anal. Biochem. 4, 330, 1962) shows an HY acid content of 78.8% in weight.

Example 31 - Preparation of the Salt of a Hyaluronic Acid (HY) with Gentamicin

1.45 g of gentamicin sulfate (10 mEq) are solubilized in 25 ml of distilled H₂O. The solution is eluted in a thermostatic column at 5°C, containing 15 ml of quaternary ammonium resin (Dowex 1x8) in OH⁻ form. The eluate, free from sulfates, is gathered in a thermostatic container at 5°C.

4.0 g of sodium salt of HY with a molecular weight of 170,000 (corresponding to 10 mEq of a monomeric unit) are solubilized in 400 ml of distilled H₂O. The solution is then eluted in a thermostatic column at 5°C, containing 15 ml of sulfonic resin (Dowex 50x8) in H⁺ form. The eluate, free from sodium, is gathered under agitation in a vortex in the solution of gentamicin base. The thick and very viscous precipitate which form is separated by decantation and freeze-dried. Yield: 4.65 g. In the salt thus obtained, all the HY acid groups are salified with gentamicin.

Quantitative microbiological determination carried out on *S. epidermidus* ATCC 12228 compared to standard gentamicin shows a content in weight of 20.0% of gentamicin base, corresponding to the theoretical content. Colorimetric determination of the glucuronic acid combined in polysaccharide according to the method of Bitter et al. (Anal. Biochem. 4, 330, 1962) shows an HY acid content of 80.0%.

Example 32 - Preparation of the Salt of a Hyaluronic Acid (HY) with Amikacin

1.47 g of amikacin sulfate (10 mEq) are solubilized in 100 ml of distilled H₂O at 5°C.

4.0 g of sodium salt of HY with a molecular weight of 170,000 (corresponding to 10 mEq of a monomeric unit) are solubilized in 400 ml of distilled H₂O. The solution is then eluted in a thermostatic column at 5°C, containing 15 ml of sulfonic resin (Dowex 50x8) in H⁺ form.

The eluate, free from sodium, is gathered under agitation in a vortex in the solution of amikacin base.

The thick and extremely viscous precipitate which forms is separated by decantation and freeze-dried. Yield: 5.16 g.

In the salt thus obtained, all the HY acid groups are salified with amikacin.

Quantitative microbiological determination carried out on *St. aureus* ATCC 29737 in comparison to standard amikacin shows a content of 27.7% in weight in amikacin base, corresponding to the theoretical content. Colorimetric determination of the glucuronic acid combined in polysaccharide according to the method of Bitter et al. (Anal. Biochem. 4, 330, 1962) shows an HY acid content of 72.3% in weight.

Example 33 - Preparation of the Salt of a Hyaluronic Acid (HY) with Rolitetracycline

4.0 g of HY sodium salt with a molecular weight of 170,000 (corresponding to 10 mEq of a monomeric unit) are solubilized in 400 ml of distilled H₂O. The solution is then eluted in a thermostatic column at 5°C, containing 15 ml of sulfonic resin (Dowex 50x8) in H⁺ form. The eluate, free from sodium, is kept at a temperature of 5°C.

5.3 g of rolitetracycline base (10 mEq) are added to the solution of HY acid under agitation at 5°C, away from the light, until complete solubilization has been achieved. The solution thus obtained is instantly frozen and freeze-dried. Yield: 8.9 g. In the salt thus obtained, all the HY acid groups are salified with rolitetracycline.

Microbiological determination on *B. pumilus* ATCC 14884 in comparison to standard rolitetracycline shows a content of 58.2% in weight in rolitetracycline base, corresponding to the theoretical value. Colorimetric determination of the glucuronic acid combined in polysaccharide according to the method of Bitter et al. (Anal. Biochem. 4, 330, 1962) shows an HY acid content of 41.8% in weight.

Example 34 - Preparation of the Salt of a Hyaluronic Acid (HY) with Polymyxin B

2.4 g of polymyxin B base (10 mEq) are suspended in
5 100 ml of distilled H₂O at 5°C

4.0 g of HY sodium salt with a molecular weight of 170,000 (corresponding to 10 mEq of a monomeric unit) are solubilized in 400 ml of distilled H₂O. The solution is then eluted in a thermostatic column at 5°C, containing 15 ml of sulfonic resin (Dowex 50x8) in H⁺ form. The eluate, free from sodium, is gathered under vigorous agitation in the suspension of polymyxin base at 5°C. After an initial phase during which the solution becomes clear, there is a progressive formation of a not
10 easily soluble product which is completely precipitated by 5 volumes of acetone. The precipitate is filtered, washed with acetone and then vacuum dried. Yield: 6.05 g. In the salt thus obtained, all the HY acid groups are salified with polymyxin B.

20 Quantitative microbiological determination carried out on *B. bronchiseptica* ATCC 4617 in comparison to standard polymyxin B shows a content of 38.7% in polymyxin B base, corresponding to the theoretical value. Colorimetric determination of the glucuronic
25 acid combined in polysaccharide according to the method of Bitter et al. (Anal. Biochem. 4, 330, 1962) shows an HY acid content of 61.3%.

Example 35 - Preparation of the Salt of a Hyaluronic Acid (HY) with Gramicidin S

6.7 g of gramicidin S hydrochloride (10 mEq) are suspended in 200 ml of ethanol/H₂O, 80:20, v/v. The solution is then eluted in a thermostatic column at 5°C, containing 15 ml of quaternary ammonium resin (Dowex
35 1x8) in OH⁻ form.

4.0 g of the sodium salt of HY with a molecular weight of 165,000 (corresponding to 10 mEq of a monomeric unit) are solubilized in 400 ml of distilled H₂O. The solution is then eluted in a thermostatic column at 5°C, containing 15 ml of sulfonic resin (Dowex 50x8) in H⁺ form. 200 ml of DMSO are added to the eluate, free from sodium, and the mixture is kept under agitation at 5°C. The solution of gramicidin base is then slowly added. The resulting solution is precipitated by 10 volumes of acetone. The precipitate is filtered, washed with acetone and vacuum dried. Yield: 9.55 g. In the salt thus obtained, all the HY acid groups are salified with gramicidin S.

Quantitative microbiological determination carried out on *S. faecium* ATCC 10541 in comparison to standard gramicidin S shows a content of 60.0% of gramicidin S base, corresponding to the theoretically value. Colorimetric determination of the glucuronic acid combined in polysaccharide according to the method of Bitter et al. (Anal. Biochem. 4, 330, 1962) shows an HY acid content of 40.0%.

Example 36 - Preparation of the Salt of a Hyaluronic Acid (HY) with Neomycin and with Polymyxin

4.0 g of the HY sodium salt with a molecular weight of 170,000 (corresponding to 10 mEq of a monomeric unit) are solubilized in 400 ml of distilled H₂O. The solution is eluted in a thermostatic column at 5°C, containing 15 ml of sulfonic resin (Dowex 50x8) in H⁺ form. The eluate, free from sodium, is gathered in a thermostatic container at 5°C. 0.150 g of Polymyxin B base (0.63 mEq) are added under vigorous agitation. 1.425 g of Neomycin sulphate (9.37 mEq) are solubilized in 25 ml of distilled H₂O. The solution is eluted in a thermostatic column at 5°C, containing 15 ml of

quaternary ammonium resin (Dowex 1x8) in OH^- form.

The eluate, free from sulphates, is gathered under vigorous agitation in the solution of HY acid and Polymyxin B. The precipitate which forms is separated by centrifugation and vacuum dried. Yield: 4.85 g. 17.25 mg of this product contain:

NEOMYCIN equal to 5.0 mg of NEOMYCIN SULPHATE

POLYMYXIN B equal to 0.63 mg (about 5000 UI) of POLYMYXIN SULPHATE

N.B. Determinations were carried out after separation by HPLC of the two active principles.

Example 37 - Preparation of the Salt of a Hyaluronic Acid (HY) with Streptomycin and with Sodium

98.68 g of HY sodium salt with a molecular weight of 255,000 (corresponding to 246 mEq of a monomeric unit) are solubilized in 8.5 l of distilled H_2O . The solution is then eluted in a thermostatic column at 5°C , containing 300 ml of sulfonic resin (Dowex 50x8) in H^+ form. The eluate, free from sodium, is gathered in a thermostatic container at 5°C .

1.88 g of Streptomycin sulphate (7.74 mEq) are solubilized in 20 ml of distilled H_2O . The solution is then eluted in a thermostatic column at 5°C , containing 12 ml of quaternary ammonium resin (Dowex 1x8) in OH^- form. The eluate, free from sulphates, is gathered under agitation in the solution of HY acid. 238.3 ml of a solution of 1 M NaOH are slowly added under agitation and the resulting solution is instantly frozen and freeze-dried. Yield: 99.8 g. 100 g of the product contain 1.5 g of Streptomycin as a base.

EXAMPLE 38

A paste comprising the 25% benzyl ester of hyaluronic acid, HYAFF 11p25, having a molecular weight between 160,000 and 230,000 Daltons, and granules of hydroxyapatite with a nominal diameter of 420 to 1,000 μ m, was obtained in the following manner.

5 grams of HYAFF 11p25, sterilized using gamma rays at an intensity of 1.25 Mrad, were added to 50 grams of sterilized water. The system was treated in a double spiral mixer at 40 rpm. The temperature was kept at 30°C \pm 2°C, and solubilization was performed for 8 hours. The solution was then placed in a high vacuum for 2 hours at 0.01 mbar to eliminate the dissolved air. The resulting viscosity was 23 Pa.s.

Subsequently, to 1 gr of solution was added 0.5 gr of commercially available hydroxyapatite ("INTERPORE200") and the mixture was stirred by hand with a spatula until a homogeneous paste was obtained. The granules proved to be bound together, forming a material which was easy to insert into cavities without the risk of the granular bone replacement becoming dislodged, either during or after the operation.

EXAMPLE 39

A paste comprising the 50% ethyl ester of hyaluronic acid, HYAFF 7p50, with a molecular weight of between 140,000 and 210,000 Daltons, and granules of hydroxyapatite with a nominal diameter of 420 to 1000 μ m, was obtained as follows.

7 grams of HYAFF 7p50, sterilized using gamma rays at an intensity of 1.25 Mrad, were added to 50 grams of sterilized water. The system was treated in a double spiral mixer at a speed of 40 rpm. The temperature was kept at 40°C \pm 2°C, and solubilization was performed for 8 hours. After solubilization, the solution was placed

in a vacuum at 0.01 mbar for 2 hours to eliminate any dissolved air. The resulting viscosity was 25 Pa.s.

Subsequently, to 1 gr of this solution was added 0.4 gr of commercially available hydroxyapatite ("INTERPORE200"), and the mixture was stirred by hand with a spatula until a homogeneous paste was obtained. The granules proved to be bound together, forming a material which was easy to insert into cavities without the risk of the granular bone replacement becoming dislodged, either during or after the operation.

EXAMPLE 40

A paste comprising a mixture of 50% benzyl esters of hyaluronic acid, HYAFF 11p50, with a molecular weight between 140,000 and 250,000 Daltons, and granules of porous calcium carbonate with a pore size of 630 μm to 1000 μm , was obtained as follows.

6 grams of HYAFF 11p50, sterilized using gamma rays at an intensity of 1.25 Mrad, were added to 50 grams of sterilized water. The system was treated in a double spiral mixer at a speed of 40 rpm. The temperature was kept at $40^{\circ}\text{C} \pm 2^{\circ}\text{C}$, and solubilization, was performed for 6 hours. After solubilization, the solution was maintained for 2 hours in a high vacuum at 0.01 mbar to eliminate any air. The resulting viscosity was 21 Pa.s.

Subsequently, to 1 gr of solution was added 0.6 gr of commercially available calcium carbonate ("BIOCORAL 1000"), and this was stirred by hand with a spatula until a homogeneous paste was obtained. The granules proved to be bound together, forming a material which was easy to insert into cavities without the risk of the granular bone replacement becoming dislodged, either during or after the operation.

EXAMPLE 41

A paste containing hyaluronic acid with a molecular weight of 140,000 - 180,000 Daltons and granules of hydroxyapatite with a nominal diameter size of 420-1000 μm was obtained in the following manner:

7 gr of hyaluronic acid, sterilized by filtration, were added to 50 gr of sterilized water. The system was treated in a double spiral mixer at 40 rpm. The temperature was kept at 25°C and solubilization was performed for 16 hours. the solution was then placed under high vacuum for 2 hours to eliminate the dissolved air. The resulting viscosity was 24.7 Pa.s.

Subsequently, to 1 gr of solution was added 0.4 gr of commercially available hydroxyapatite (Interpore 200) and the mixture was stirred by hand with a spatula until a homogeneous paste was obtained. The granules proved to be bound together, forming a material which was easy to insert into cavities without the risk of the granular bone replacement becoming dislodged, either during or after the operation.

Animal Model and Treatment

In order to enhance retention of hydroxyapatite particles and to promote ease of handling, the above-mentioned preparation was used to fill fresh tooth extraction sites in canines. Beagle dogs were used as the animal model. Maxillary and mandibular incisors and premolars were removed, so that each animal received 12 defects, six in the maxilla, six in the mandible. Subsequently, the interradicular septum was removed to increase the volume of the defect. The extraction sites were implanted with the above-mentioned preparation, left empty, or filled with hydroxyapatite granules as controls. Simple suturing was used to bring the gingival flaps together. Animals were sacrificed 1, 2

and 3 months after surgery. At the time of sacrifice, tissue fixation was performed using the perfusion technique through incannulation of the maxillary artery and injection of Karnowsky fixative.

5 This study showed the ease of use of hyaluronic acid-hydroxyapatite composites to fill fresh tooth extraction sites. The mixture can be manually inserted or injected under pressure into a bone defect *in situ*. Retention of particles was achieved; with time, the
10 bonding solution was resorbed, and bone ingrowth progressed.

 The invention being thus described, it will be obvious that the same may be varied in many ways. Such variations are not to be regarded as a departure from
15 the spirit and scope of the invention, and all such modifications as would be obvious to one skilled in the art are intended to be included within the scope of the following claims.

CLAIMS:

- 1 1. A bonding solution for granular bone
2 replacements, comprising at least one member selected
3 from the group consisting of hyaluronic acid and a
4 hyaluronic acid derivative.
- 1 2. The bonding solution of claim 1, wherein said
2 hyaluronic acid derivative is a hyaluronic acid ester.
- 1 3. The bonding solution of claim 1, wherein said
2 hyaluronic acid derivative is a salt of hyaluronic acid
3 in association with an antibiotic.
- 1 4. The bonding solution of claim 1, wherein said
2 solution has a viscosity in the range of from about 15
3 Pa.s to about 30 Pa.s at a temperature of 25°C and
4 approximately 50% relative humidity.
- 1 5. The bonding solution of claim 1, wherein said
2 solution has a viscosity in the range of from about 22
3 Pa.s to about 25 Pa.s at a temperature of 25°C and
4 approximately 50% relative humidity.

1 6. The bonding solution of claim 2, wherein said
2 hyaluronic acid ester is at least one member selected
3 from the group consisting of the 25% benzyl ester of
4 hyaluronic acid, the 50% benzyl ester of hyaluronic
5 acid, and the 50% ethyl ester of hyaluronic acid.

1 7. A paste for granular bone replacements,
2 comprising the bonding solution of claim 1 and natural
3 or artificial bone granules.

1 8. The paste of claim 7, wherein said bonding
2 solution and said bone granules are present in a ratio
3 of about 1:3, w/w.

1 9. The paste of claim 7, wherein the diameter of
2 said bone granules is in the range of from about 50 μm
3 to about 5 mm.

1 10. The paste of claim 7, wherein said bone
2 granules are porous or non-porous.

1 11. The paste of claim 7, wherein said bone
2 granules are selected from the group consisting of
3 hydroxyapatite, tricalcium phosphate, and calcium
4 carbonate.

1 12. The paste of claim 7, comprising a solution of
2 the 25% benzyl ester of hyaluronic acid having a
3 molecular weight between about 160,000 Daltons and about
4 230,000 Daltons, and granules of hydroxyapatite having
5 a diameter of from about 420 μm to about 1,000 μm .

1 13. The paste of claim 7, comprising a solution of
2 the 50% ethyl ester of hyaluronic acid having a
3 molecular weight between about 140,000 Daltons and about
4 210,000 Daltons, and granules of hydroxyapatite having
5 a diameter of from about 420 μm to about 1,000 μm .

1 14. The paste of claim 7, comprising a solution of
2 the 50% benzyl ester of hyaluronic acid having a
3 molecular weight between about 140,000 Daltons and about
4 250,000 Daltons, and granules of porous calcium
5 carbonate having a diameter of from about 630 μm to
6 about 1,000 μm .

1 15. A method of promoting the growth or repair of
2 damaged or defective bone in human or veterinary
3 dentistry or surgery, comprising contacting said damaged
4 or defective bone with an effective amount of said paste
5 of claim 7.

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all) ⁶ According to International Patent Classification (IPC) or to both National Classification and IPC Int.Cl. 5 A61L27/00														
II. FIELDS SEARCHED <div style="text-align: center; border: 1px solid black; padding: 2px; margin: 5px 0;">Minimum Documentation Searched⁷</div> <table border="1" style="width: 100%; border-collapse: collapse;"> <tr> <th style="width: 30%; padding: 5px;">Classification System</th> <th style="padding: 5px;">Classification Symbols</th> </tr> <tr> <td style="padding: 5px;">Int.Cl. 5</td> <td style="padding: 5px;">A61L</td> </tr> </table> <div style="text-align: center; border: 1px solid black; padding: 2px; margin: 5px 0;">Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched⁸</div>			Classification System	Classification Symbols	Int.Cl. 5	A61L								
Classification System	Classification Symbols													
Int.Cl. 5	A61L													
III. DOCUMENTS CONSIDERED TO BE RELEVANT⁹ <table border="1" style="width: 100%; border-collapse: collapse;"> <thead> <tr> <th style="width: 10%; padding: 5px;">Category¹⁰</th> <th style="width: 70%; padding: 5px;">Citation of Document,¹¹ with indication, where appropriate, of the relevant passages¹²</th> <th style="width: 20%; padding: 5px;">Relevant to Claim No.¹³</th> </tr> </thead> <tbody> <tr> <td style="text-align: center; vertical-align: top; padding: 5px;">X</td> <td style="padding: 5px;"> WO,A,9 117 777 (UNIVERSITY OF FLORIDA) 28 November 1991 see page 12, line 5; claims 1,4,5,17,24 <div style="text-align: center;">---</div> </td> <td style="text-align: center; vertical-align: top; padding: 5px;"> 1,3, 7-11,15 </td> </tr> <tr> <td style="text-align: center; vertical-align: top; padding: 5px;">X</td> <td style="padding: 5px;"> DATABASE WPIL Week 8844, Derwent Publications Ltd., London, GB; AN 88-3114711 & JP,A,63 229 058 (OSHIMA) see abstract <div style="text-align: center;">----</div> </td> <td style="text-align: center; vertical-align: top; padding: 5px;"> 1,7,11, 15 </td> </tr> <tr> <td style="text-align: center; vertical-align: top; padding: 5px;">P,X</td> <td style="padding: 5px;"> EP,A,0 522 569 (UNITED STATES SURGICAL CORPORATION) 13 January 1993 see page 4, line 13 - line 17; claims 1,9,17; examples 7,8 <div style="text-align: center;">-----</div> </td> <td style="text-align: center; vertical-align: top; padding: 5px;"> 1-3,7, 9-11,15 </td> </tr> </tbody> </table>			Category ¹⁰	Citation of Document, ¹¹ with indication, where appropriate, of the relevant passages ¹²	Relevant to Claim No. ¹³	X	WO,A,9 117 777 (UNIVERSITY OF FLORIDA) 28 November 1991 see page 12, line 5; claims 1,4,5,17,24 <div style="text-align: center;">---</div>	1,3, 7-11,15	X	DATABASE WPIL Week 8844, Derwent Publications Ltd., London, GB; AN 88-3114711 & JP,A,63 229 058 (OSHIMA) see abstract <div style="text-align: center;">----</div>	1,7,11, 15	P,X	EP,A,0 522 569 (UNITED STATES SURGICAL CORPORATION) 13 January 1993 see page 4, line 13 - line 17; claims 1,9,17; examples 7,8 <div style="text-align: center;">-----</div>	1-3,7, 9-11,15
Category ¹⁰	Citation of Document, ¹¹ with indication, where appropriate, of the relevant passages ¹²	Relevant to Claim No. ¹³												
X	WO,A,9 117 777 (UNIVERSITY OF FLORIDA) 28 November 1991 see page 12, line 5; claims 1,4,5,17,24 <div style="text-align: center;">---</div>	1,3, 7-11,15												
X	DATABASE WPIL Week 8844, Derwent Publications Ltd., London, GB; AN 88-3114711 & JP,A,63 229 058 (OSHIMA) see abstract <div style="text-align: center;">----</div>	1,7,11, 15												
P,X	EP,A,0 522 569 (UNITED STATES SURGICAL CORPORATION) 13 January 1993 see page 4, line 13 - line 17; claims 1,9,17; examples 7,8 <div style="text-align: center;">-----</div>	1-3,7, 9-11,15												
<div style="display: flex; justify-content: space-between;"> <div style="width: 45%;"> <p>¹⁰ Special categories of cited documents:</p> <p>"A" document defining the general state of the art which is not considered to be of particular relevance</p> <p>"E" earlier document but published on or after the international filing date</p> <p>"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)</p> <p>"O" document referring to an oral disclosure, use, exhibition or other means</p> <p>"P" document published prior to the international filing date but later than the priority date claimed</p> </div> <div style="width: 45%;"> <p>"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention</p> <p>"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step</p> <p>"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.</p> <p>"A" document member of the same patent family</p> </div> </div>														
IV. CERTIFICATION <table border="1" style="width: 100%; border-collapse: collapse;"> <tr> <td style="width: 50%; padding: 5px;"> Date of the Actual Completion of the International Search <div style="text-align: center; font-weight: bold;">10 AUGUST 1993</div> </td> <td style="width: 50%; padding: 5px;"> Date of Mailing of this International Search Report <div style="text-align: center; font-weight: bold;">20.08.93</div> </td> </tr> <tr> <td style="padding: 5px;"> International Searching Authority <div style="text-align: center; font-weight: bold;">EUROPEAN PATENT OFFICE</div> </td> <td style="padding: 5px;"> Signature of Authorized Officer <div style="text-align: center; font-weight: bold;">PELTRE CHR.</div> </td> </tr> </table>			Date of the Actual Completion of the International Search <div style="text-align: center; font-weight: bold;">10 AUGUST 1993</div>	Date of Mailing of this International Search Report <div style="text-align: center; font-weight: bold;">20.08.93</div>	International Searching Authority <div style="text-align: center; font-weight: bold;">EUROPEAN PATENT OFFICE</div>	Signature of Authorized Officer <div style="text-align: center; font-weight: bold;">PELTRE CHR.</div>								
Date of the Actual Completion of the International Search <div style="text-align: center; font-weight: bold;">10 AUGUST 1993</div>	Date of Mailing of this International Search Report <div style="text-align: center; font-weight: bold;">20.08.93</div>													
International Searching Authority <div style="text-align: center; font-weight: bold;">EUROPEAN PATENT OFFICE</div>	Signature of Authorized Officer <div style="text-align: center; font-weight: bold;">PELTRE CHR.</div>													

**ANNEX TO THE INTERNATIONAL SEARCH REPORT
ON INTERNATIONAL PATENT APPLICATION NO.**

EP 9300933
SA 73984

This annex lists the patent family members relating to the patent documents cited in the above-mentioned international search report.
The members are as contained in the European Patent Office EDP file on
The European Patent Office is in no way liable for these particulars which are merely given for the purpose of information.

10/08/93

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO-A-9117777	28-11-91	None	
EP-A-0522569	13-01-93	None	

**This Page is Inserted by IFW Indexing and Scanning
Operations and is not part of the Official Record**

BEST AVAILABLE IMAGES

Defective images within this document are accurate representations of the original documents submitted by the applicant.

Defects in the images include but are not limited to the items checked:

- ☐ BLACK BORDERS
- ☐ IMAGE CUT OFF AT TOP, BOTTOM OR SIDES
- ☒ FADED TEXT OR DRAWING
- ☒ BLURRED OR ILLEGIBLE TEXT OR DRAWING
- ☐ SKEWED/SLANTED IMAGES
- ☐ COLOR OR BLACK AND WHITE PHOTOGRAPHS
- ☐ GRAY SCALE DOCUMENTS
- ☐ LINES OR MARKS ON ORIGINAL DOCUMENT
- ☐ REFERENCE(S) OR EXHIBIT(S) SUBMITTED ARE POOR QUALITY
- ☐ OTHER: _____

IMAGES ARE BEST AVAILABLE COPY.

As rescanning these documents will not correct the image problems checked, please do not report these problems to the IFW Image Problem Mailbox.